Gangliosides Inhibit Urokinase-Type Plasminogen Activator (uPA)-Dependent Squamous Carcinoma Cell Migration by Preventing uPA Receptor/$\alpha_5\beta_1$ Integrin/Epidermal Growth Factor Receptor Interactions

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The interaction of the urokinase-type plasminogen activator (uPA) receptor (uPAR) with integrins plays a critical role in the regulation of cell adhesion and migration. However, the molecular events underlying the modulation of the interaction of uPAR and integrin are poorly understood. Gangliosides are thought to regulate epithelial cell adhesion and migration by inhibiting $\alpha_5\beta_1$ integrin and epidermal growth factor receptor (EGFR) signaling. We report here that increases in the expression of ganglioside NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GT1b) or NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GM3) inhibit uPA-dependent cell migration by preventing the association of uPAR with $\alpha_5\beta_1$ integrin or uPAR/$\alpha_5\beta_1$ integrin with the EGFR, respectively. As a result, uPA-dependent focal adhesion kinase (FAK) and integrin-mediated EGFR signaling are suppressed. Both gangliosides inhibit uPAR signaling-stimulated migration; however, GM3 inhibits uPA-induced EGFR phosphorylation by blocking the crosstalk between integrin and EGFR, whereas GT1b suppresses both uPA-induced FAK and EGFR activation by preventing the activation of integrin $\alpha_5\beta_1$.

Key words: antisense/FAK/GPI/PPPP/sialidase
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Epithelial cell motility plays a fundamental role in both physiologic and pathologic conditions, such as tissue resorption and remodeling during embryogenesis, wound healing, and metastasis of carcinomas. The mechanisms involved in the modulation of cell motility, however, are poorly understood, especially at the membrane level. The coordinated expression and function of adhesion receptors (particularly integrins) and of the protease systems that counteract cell–substratum and cell–cell interactions play a critical role. One of these protease systems, the plasminogen activation system, is comprised of plasminogen, the plasminogen activators (urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator), their respective cell surface receptors, and their inhibitors (Chapman, 1997). The uPA receptor (uPAR), a glycosylphosphatidylinositol (GPI)-anchored glycoprotein located in the outer leaflet of the plasma membrane, has been shown to initiate signal transduction events and affects cell proliferation, adhesion, migration, and invasion (Chapman, 1997; Xue *et al*, 1997; Aguirre Ghiso *et al*, 1999; Ossowski *et al*, 1999; Andreasen *et al*, 2000; Ossowski and Aguirre-Ghiso, 2000; Aguirre-Ghiso *et al*, 2001; Ma *et al*, 2001; Jo *et al*, 2002). As such, uPAR signaling is thought to participate in cell migration during development, wound healing, vascular remodeling, and cancer cell metastasis and invasion. The activation and/or expression of uPAR on cells strongly correlate with their migratory and invasive potential (Ossowski *et al*, 1991; Aguirre Ghiso *et al*, 1999), and are able to be modulated by several factors, including growth factors (epidermal growth factor (EGF), transforming growth factor-β, etc.), cytokines (interleukin-1, interleukin-8, etc.), and matrix molecules, particularly vitronectin (Wei *et al*, 1994; Yebra *et al*, 1999; Sandberg *et al*, 2001; Shiratsuchi *et al*, 2002; Unlu and Leake, 2003). uPAR is also able to trigger activation of several signaling pathways and thus to modulate cell adhesion and migration indirectly, such as via activation of $\alpha_5\beta_1$ integrin and focal adhesion kinase (FAK) signaling when cells grow on a fibronectin matrix (Yebra *et al*, 1999; Aguirre Ghiso, 2002).

Lacking both transmembrane and intracytoplasmic domains (Ploug *et al*, 1991), uPAR-modulated signaling transduction is thought to require transmembrane adaptors (Resnati *et al*, 1996), particularly integrins (Wei *et al*, 1996; Tarui *et al*, 2001, 2003; Kugler *et al*, 2003), and EGF receptor

Abbreviations: EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; GD2, GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer; GD3, NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer; GM2, GalNAcβ1 → 4(NeuAcα2 → 3)Gal β1 → 4Glcβ1-Cer; GM3, NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer; GPI, glycosylphosphatidylinositol; GT1b, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer; PPPP, *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl; RU-486, mifepristone; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor

(EGFR) (Liu *et al*, 2002; Jo *et al*, 2003). uPAR has been shown to associate with integrins based on co-immunoprecipitation, co-immunolocalization, and resonance energy transfer studies (Xue *et al*, 1994, 1997; Wei *et al*, 1996; Kugler *et al*, 2003). uPAR may also act as the primary ligand for $\alpha_5\beta_1$ integrin upon uPA-induced cell adhesion and migration (Tarui *et al*, 2001, 2003; Kugler *et al*, 2003). In this way, uPA/uPAR signal transduction is initiated through binding to functional $\alpha_5\beta_1$ integrin. uPAR also appears to form a complex with EGFR to facilitate EGFR signaling, thus activating several kinases, including mitogen-activated protein kinases, phosphatidylinositol-3-kinase, and Src kinases. Through its effect on EGFR signaling, uPA is able to modulate cell proliferation, adhesion, migration, invasion, and survival (Della Rocca *et al*, 1997, 1999; Prenzel *et al*, 2001; Liu *et al*, 2002). The mechanism of the role of transmembrane receptors, including integrin and EGFR, in uPA/uPAR signaling is not fully established.

Gangliosides, sialylated glycosphingolipids that are located on the outer leaflet of the plasma cell membrane, are thought to participate in the regulation of several biological processes, including cell proliferation, adhesion, migration, differentiation, and apoptosis (Paller *et al*, 1995; Sung *et al*, 1998; Wang *et al*, 2001a, b, c, 2002a, b, 2003a; Sun *et al*, 2002). Gangliosides are distinguished from one another by the extent and location of their sialic acid residues, as well as by their carbohydrate structure linked to a ceramide core. Our studies have shown that NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GM3), the predominant ganglioside of epithelial cells (Wang *et al*, 2001b), interacts directly with EGFR (Wang *et al*, 2001d) and inhibits both ligand-dependent and ligand-independent activation of EGFR (Wang *et al*, 2001a, d, 2002a, 2003a), thus inhibiting cell proliferation (Wang *et al*, 2001a, 2003a), migration, and invasion (Wang *et al*, 2003b). The highly sialylated ganglioside, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GT1b), strongly inhibits adhesion, spreading, and migration of cultured epithelial cells, specifically when cells are plated on a fibronectin matrix (Paller *et al*, 1995; Sung *et al*, 1998; Wang *et al*, 2001b, 2002b). GT1b binds directly to the α_5 subunit of $\alpha_5\beta_1$ integrin (Wang *et al*, 2001b) and inhibits its activation and downstream signaling, including FAK, c-Src, phosphatidylinositol-3-kinase (Wang *et al*, 2002b), Ras,[1] and integrin-linked kinase (Wang *et al*, 2001c), to prevent cell adhesion, impair motility (Paller *et al*, 1995; Wang *et al*, 2001b, 2002b), and trigger cell apoptosis (Wang *et al*, 2001c).

We have evaluated the effects of endogenous and exogenous modulation of gangliosides GT1b and GM3 on the motility of squamous carcinoma cells, and have addressed the possibility that ganglioside expression modulates uPAR signaling. These studies show that alterations in GT1b and GM3 expression differentially affect uPAR-directed signaling in this carcinoma cell line at the membrane level. GT1b modulates the ability of uPAR to form a complex with $\alpha_5\beta_1$ integrin and thereby prevents the activation of $\alpha_5\beta_1$ integrin in the face of uPA, which results in the disassociation of uPAR/$\alpha_5\beta_1$ integrin with EGFR, whereas GM3 regulates the crosstalk of uPAR/$\alpha_5\beta_1$ integrin with EGFR. Although both gangliosides inhibit uPA-induced cell migration, GT1b prevents uPAR signaling-directed FAK and EGFR activation and totally inhibits cell migration in the face of uPA, whereas GM3 inhibits uPAR signaling-stimulated EGFR phosphorylation and only partially reverses cell migration in the face of uPA.

[1]Wang XQ, Sun P, Paller AS: Ganglioside modulated keratinocyte adhesion, spreading and migration by both integrin-and EGFR-mediated signaling pathways. J Invest Dermatol 117:474, 2001 (abstr).

Results

Ganglioside modulation regulates cell migration We have used a variety of genetic and biochemical manipulations to change specific ganglioside expression in SCC12 squamous carcinoma cells (Fig 1, Table I). When the expression of ganglioside is manipulated, a significant difference in migration is first noted within 12 h of culture in the face of uPA (Fig 2). Ganglioside depletion by either *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl (PPPP) treatment (Fig 2*A*, the *3rd bar*, $p < 0.001$) or sialidase overexpression (Fig 2*B*, the *3rd bar*, $p < 0.001$) leads to a 3.1–3.8-fold increase in cell migration by both scratch assay (not shown) and chemotaxis migration assay (Fig 2) in

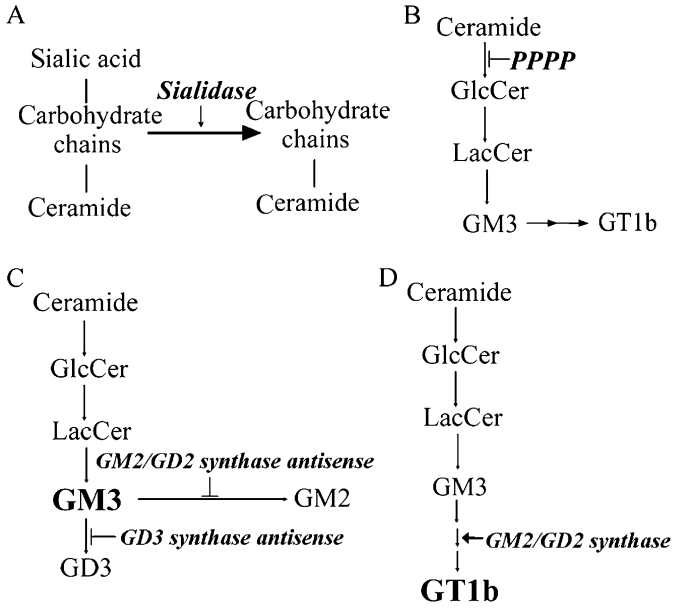

Figure 1
Endogenous modulation of gangliosides NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GM3) and NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GT1b) expression. Overexpression of a human plasma ganglioside-specific sialidase depletes functional gangliosides by cleaving sialic acid residue(s) from gangliosides (*A*). Incubation of SCC12 cells with *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl (PPPP) to deplete GM3 and downstream gangliosides, such as GT1b (*B*). Treatment with antisense oligodeoxynucleotides to both GalNAcβ1 → 4(NeuAcα2 → 3)Gal β1 → 4Glcβ1-Cer (GM2)/GD2 synthase and NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GD3) synthase prevents the synthesis of downstream ganglioside components GM2 and GD3, respectively, leading to endogenous overexpression of GM3 (*C*). Stable transfection of cells with a human GM2/GD2 synthase cDNA in an inducible system and induction with mifepristone (RU-486) as described (Wang *et al*, 2002b) induces GT1b overexpression in SCC12 cells (*D*).

Table I. Results of ganglioside modulation (Wang *et al*, 2001c, 2002a, b; Sun *et al*, 2002)

Cell line or treatment	Ganglioside expression	Control(s) (ganglioside expression of parental SCC12 cells)
SCC12 cells transfected with plasma ganglioside-specific *sialidase* cDNA ("SSIA")	GM3 ↓↓ GT1b ↓↓	Vector transfection (*pcDNA*)
SCC12 cells treated with *PPPP*	GM3 ↓↓ GT1b ↓↓	Vehicle (DMSO)-treated cells
SCC12 cells treated with oligodeoxynucleotides directed against both *GM2/GD2 synthase* and *GD3 synthase*	GM3 ↑↑ GT1b ↓↓	Sense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase
SCC12 cells treated with *purified GM3*	GM3 ↑↑	Vehicle (DMSO)-treated cells
		Ganglioside GM2-treated cells
SCC12 cells treated with *PPPP* and "rescued" with *purified GM3*	GM3 ↑↑ GT1b ↓↓	SCC12 cells treated with PPPP and "rescued" with control ganglioside GM2
SCC12 cells treated with PPPP and "rescued" with *purified GT1b*	GM3 ↓↓ GT1b ↑↑	
SCC12 cells transfected with *GM2/GD2 synthase* gene and induced with RU486	GT1b ↑↑	Vector transfection *(p17x4-GLVP)*
		GM2/GD2 synthase transfection without RU486 induction
SCC12 cells treated with *purified GT1b*	GT1b ↑↑	Vehicle (DMSO) treated cells
		Ganglioside GM2-treated cells
SCC12 cells treated with oligodeoxynucleotides directed against both *GM2/GD2 synthase* and *GD3 synthase*, "rescued" with *purified GT1b*	GM3 ↑↑ GT1b ↑↑	SCC12 cells treated with oligodeoxynucleotides directed against both GM2/GD2 synthase and GD3 synthase, "rescued" with control ganglioside GM2

PPPP, *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl; DMSO, dimethyl sulfoxide; GM3, NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer; GT1b, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer; GLVP, N=terminal VP16 transcriptional activation domain fused to a yeast GAL4 DNA binding domain; GM2, GalNAcβ1 → 4(NeuAcα2 → 3)Gal β1 → 4Glcβ1-Cer; GD2, GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer.

comparison with dimethyl sulfoxide (DMSO) vehicle-treated (*A*, the *2nd bar*), pcDNA vector-transfected (*B*, the *2nd bar*), or untreated parental SCC12 (the *1st bars of A* and *B*) controls. In the absence of uPA, migration does not occur, despite depletion of ganglioside (*bars on the right side of* Fig 2*A–F*).

Increases in GT1b expression by either overexpression of GalNAcβ1 → 4(NeuAcα2 → 3)Gal β1 → 4Glcβ1-Cer (GM2)/GD2 synthase (Fig 2*C*, the *4th bar*) or pharmacological addition of purified GT1b (Fig 2*D*, the *5th bar*) inhibit uPA-dependent cell migration by 2.5–2.6-fold in comparison with p17x4-GLVP mock vector control (*C*, the *2nd bar*), the GM2/GD2 synthase cDNA-transfected cells without RU-486 induction (*C*, the *3rd bar*), DMSO vehicle-treated cells (*D*, the *2nd bar*), cells with increased control ganglioside GM2 (*D*, the *3rd bar*), and parental SCC12 cell (the *1st bars of C* and *D*) controls ($p < 0.01$). Even in the absence of GM3, increases in GT1b expression dramatically decrease uPA-dependent cell migration (Fig 2*E*, the *4th bar*) in comparison with cells depleted of both GM3 and GT1b (*E*, the *1st bar*) and cells depleted of both GM3 and GT1b but treated with control ganglioside GM2 (*E*, the *2nd bar*) ($p < 0.001$). When both GM3 and GT1b are overexpressed by treating cells with antisense oligodeoxynucleotides to both GM2/GD2 synthase and NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GD3) synthase (GM3 ↑, GT1b ↓), but the deficient GT1b is "rescued" pharmacologically (GM3 ↑, GT1b ↑), cell migration is inhibited by 6.6-fold (Fig 2*F*, the *3rd bar*) in comparison with antisense oligodeoxynucleotides-treated cells without GT1b "rescue" (Fig 2*F*, the *1st bar*) or antisense oligodeoxynucleotides-treated cells incubated with control ganglioside GM2 (*F*, the *2nd bar*) ($p < 0.001$). When GT1b is overexpressed, the migration of cells in the absence of GM3 (Fig 2*E*, the *4th bar*) is similar to that of cells in the presence of GM3 (Fig 2*D*, the *4th bar*) and of cells that overexpress GM3 as well (Fig 2*F*, the *3rd bar*).

Increased GM3 expression by pharmacological addition of purified GM3 (Fig 2*D*, the *4th bar*) also inhibits cell migration by 1.8-fold in comparison with cells treated without (*D,* the *1st bar*) or with either DMSO vehicle control (*D,* the *2nd bar*) or control ganglioside GM2 (*D,* the *3rd bar*) ($p < 0.05$). In the absence of GT1b, pharmacologic addition of GM3 inhibits cell migration by 1.9-fold (Fig 2*E*, the *3rd bar*) in comparison with cells depleted of both GM3 and GT1b by PPPP treatment (*E*, the *1st bar*), regardless of treatment with control ganglioside GM2 (*E*, the *2nd bar*) ($p < 0.05$).

Modulation of GT1b expression affects the association of uPAR with both $\alpha_5\beta_1$ integrin and EGFR Depletion of GM3 and GT1b by either sialidase overexpression (Fig 3*A*, *lanes 3* and *4*, *middle* and *bottom rows*) or PPPP treatment (Fig 3*C*, *lane 3, middle* and *bottom rows*) increases the association of both $\alpha_5\beta_1$ integrin and EGFR with uPAR in comparison with parental SCC12 cells (Fig *3A* and *C*, *lane 1*), pcDNA vector-treated cells (Fig *3A*, *lane 2*), or DMSO vehicle-treated cells (Fig 3*C*, *lane 2*). In the absence of GT1b, GM3 overexpression by antisense oligodeoxynuc-

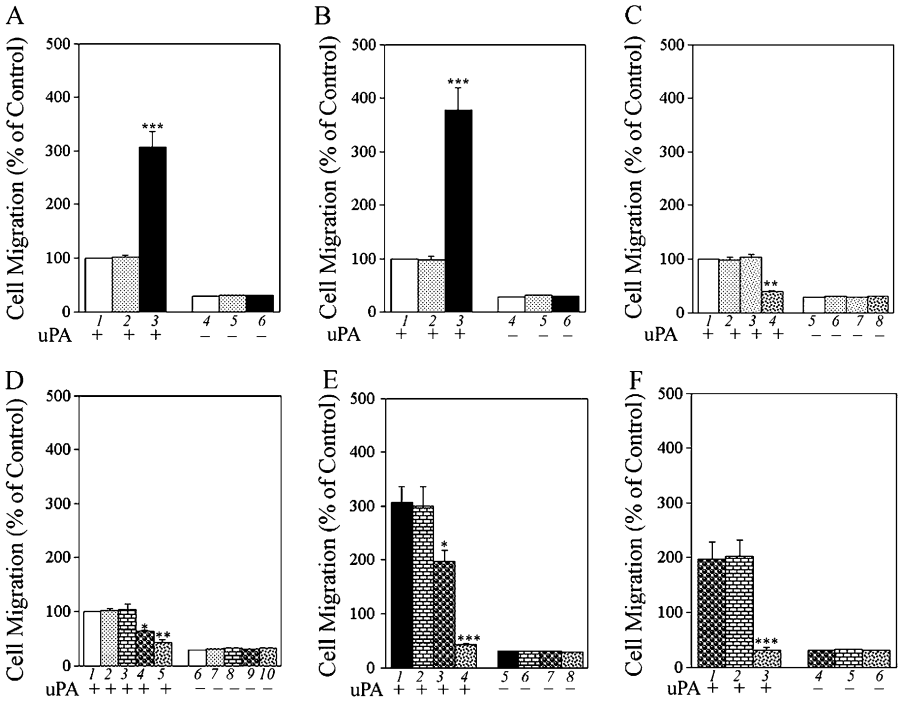

Figure 2
Ganglioside modulation regulates urokinase-type plasminogen activator- (uPA-) dependent cell migration. Cell migration was performed by both scratch assay (not shown) and chemotaxis migration assay using a Transwell system (BD Biosciences, Bedford, Massachusetts). After starvation of serum and growth factors, cells prepared as indicated below were stimulated with (+) or without (−) 10 nM of uPA. (*A*) Treatment with *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl (PPPP) (NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GM3) and NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3) Galβ1 → 4Glcβ1-Cer (GT1b) ↓); untreated SCC12 cells (*lanes 1, 4*); treated with dimethyl sulfoxide (*lanes 2, 5*); treated with PPPP (*lanes 3, 6*). (*B*) Stable transfection of SCC12 cells with human plasma ganglioside-specific sialidase (SSIA) (GM3 and GT1b ↓); SCC12 cells (*lanes 1, 4*); pcDNA mock transfected cells (*lanes 2, 5*); SSIA cells (*lanes 3, 6*). (*C*) Stable transfection of SCC12 cells with GalNAcβ1 → 4(NeuAcα2 → 3) Gal β1 → 4Glcβ1-Cer (GM2)/GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GD2) synthase and with mifepristone (RU-486) induction (GT1b ↑). Parental SCC12 cells (*lanes 1, 5*); p17x4-GLVP vector-transfected cells (*lanes 2, 6*); GM2/GD2 synthase gene-transfected cells without RU-486 induction (*lanes 3, 7*); GM2/GD2 synthase gene transfected cells with 100 nM RU-486 induction: (*lanes 4, 8*). (*D*) SCC12 cells treated with specific purified ganglioside. Parental SCC12 cells (*lanes 1, 6*); DMSO vehicle (*lanes 2, 7*); 50 μM GM2 (*lanes 3, 8*); 50 μM GM3 (*lanes 4, 9*); 1μM GT1b (*lanes 5, 10*). (*E*) PPPP-treated (ganglioside-depleted) SCC12 cells, incubated with specific purified ganglioside to "rescue" the specific ganglioside expression. SCC12 cells treated with PPPP alone (*lanes 1, 5*); + 50 μM GM2 (*lanes 2, 6*); + 50 μM GM3 (*lanes 3, 7*); + 1μM GT1b (*lanes 4, 8*). (*F*) SCC12 cells treated with antisense oligodeoxynucleotides to both GM2/GD2 synthase and NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GD3) synthase (increased GM3), incubated with purified ganglioside to "rescue" the specific ganglioside expression. SCC12 cells treated with antisense oligodeoxynucleotides alone (GM3 ↑ and GT1b ↓) (*lanes 1, 4*); + 50 μM GM2 (*lanes 2, 5*); + 1μM GT1b (GM3 ↑ and GT1b ↑) (*lanes 3, 6*). Migration was determined with a Transwell unit as indicated in Materials and Methods. The extent of migration for each cell type was determined by optical density readings at 560 nm, and expressed in comparison with SCC12 parental cells as mean percentages ± SD. Studies were performed at least three different times in duplicate. The data shown represent the mean values of 4 SSIA cell clones (SSIA3, SSIA6, SSIA12, and SSIA25), 4 GM2/GD2 synthase overexpressing clones (C-1, -5, -11, and -26), or two mock controls from each group, pcDNA (clones 1 and 3) as SSIA cell controls; p17x4-GLVP (clones 3 and 6) as controls for GM2/GD2 synthase transfection.

leotide treatment (Fig 3*A*, *lane 6, middle* and *bottom rows*) or pharmacological addition of GM3 to PPPP-treated cells (Fig 3*C*, *lane 4, middle* and *bottom rows*) leads to abrogation of the association of uPAR with EGFR, but increases the association of uPAR with $\alpha_5\beta_1$ integrin, in comparison with sense oligodeoxynucleotide-treated (*A, lane 5*), DMSO vehicle-treated (*C, lane 2*), or untreated parental SCC12 (*A* and *C, lane* 1) controls. In the presence of GT1b, GM3 overexpression by pharmacological addition of purified GM3 into SCC12 cells only blocks the association of EGFR with uPAR, without stimulating the association of $\alpha_5\beta_1$ integrin with uPAR (Fig 3*A*, *lane 14, middle* and *bottom rows*) in comparison with cells treated without (*lane 11*) or with either DMSO vehicle (*lane 12*) or ganglioside GM2 (*lane 13*) controls. Regardless of the expression of GM3, GT1b overexpression by the increased expression of GM2/GD2 synthase (Fig 3*A*, *lanes 9* and *10*) or pharmacological addition of purified GT1b (Fig 3*A*, *lane 15*; Fig 3*B*, *lanes 4–6*; Fig 3*C*, *lane 5*) disrupts the association of uPAR with $\alpha_5\beta_1$ integrin (*middle rows*), as well as the association of uPAR with the EGFR (*bottom rows*) in comparison with controls (Fig 3*A*, *lanes 1,7, 8*, and *11–13*; Fig *3B*, *lanes 1–3*; Fig 3*C*, *lanes 1–3*). Modulation of ganglioside expression does not affect the expression of uPAR, $\alpha_5\beta_1$ integrin (not shown), or EGFR (Fig 4*A*, *top row)*.

Ganglioside modulation regulates uPA-stimulated EGFR and FAK phosphorylation In the presence of uPA, depletion of gangliosides GT1b and GM3 by sialidase overexpression facilitates uPA-stimulated EGFR and FAK phosphorylation (Fig 4*A* and *B*, *lane 6, bottom rows*) in comparison with mock vector transfected cell (*lane 4*) and parental SCC12 cell (*lane 2*) controls. Depletion of GT1b in the face of increased GM3 by antisense oligodeoxynucleotide treatment stimulates uPA-induced FAK phosphorylation (Fig 4*B*, *lane 10, bottom row*), but uPA-induced EGFR phosphorylation is prevented (Fig 4*A*, *lane 10, bottom row*). Ganglioside GT1b overexpression via upregulating GM2/GD2 synthase expression inhibits uPA-induced phosphorylation of both EGFR and FAK (Fig 4*A* and *B*, *lane 16, bottom rows*). In the absence of uPA (*lanes 1, 3, 5, 7, 9, 11, 13,* and *15*), neither expression (*top row*) nor phosphorylation (*bottom row*) of EGFR (*A*) and FAK (*B*) is affected by the modulation of ganglioside expression. Ganglioside modulation has no effect on the expression of either EGFR or FAK, regardless of the absence or presence of uPA (Fig 4*A* and *B*, *top rows*).

Prevention of cell migration by gangliosides requires the inhibition of both FAK and EGFR kinase activities Inhibition of EGFR kinase activity with AG1478 or of

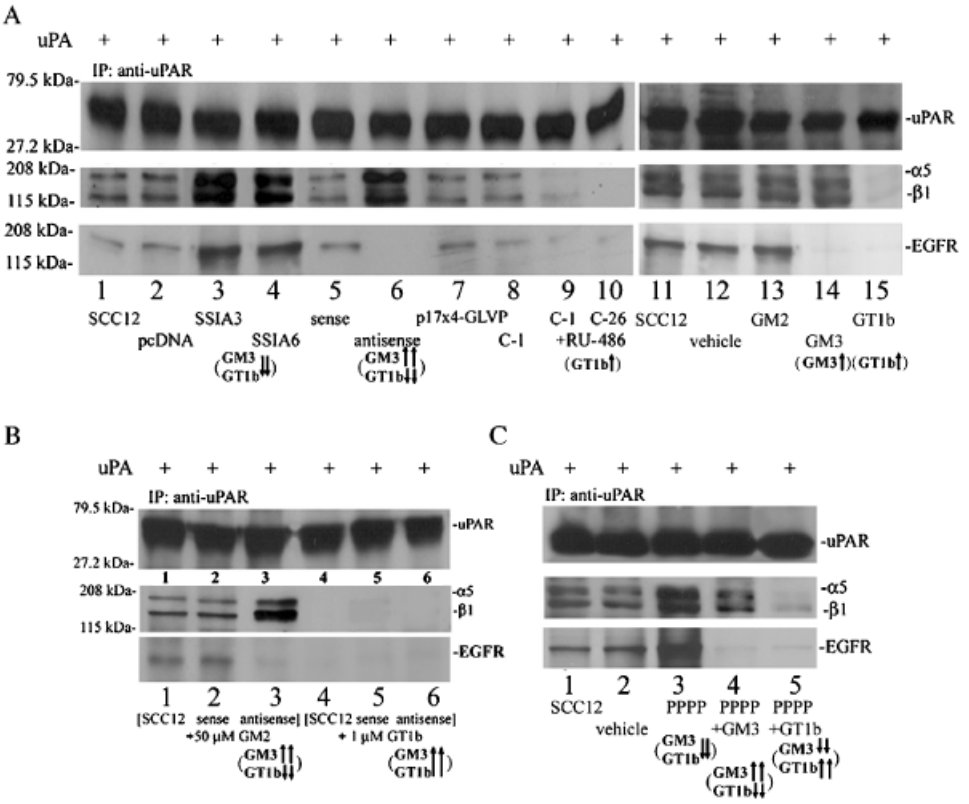

Figure 3
Alteration of NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GT1b) expression regulates the association of urokinase-type plasminogen activator receptor (uPAR) with both $\alpha_5\beta_1$ integrin and epidermal growth factor receptor (EGFR). Cells were prepared as described in *Materials and Methods* and in Fig 2. After starvation of both growth factors and serum, cells were stimulated with 10 nM urokinase-type plasminogen activator (uPA) for 10 min, and uPAR was immunoprecipitated from cells as described in *Materials and Methods*. One-third of the resulting immunoprecipitate was treated with undenatured Laemmli buffer for 30 min at room temperature and applied to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mini-gel for immunoblotting with anti-uPAR antibody to ensure equal loading (*top*); the other two-thirds of each immunoprecipitate was boiled in Laemmli buffer for 10 min, and applied to an 8% SDS-PAGE mini-gel for immunoblotting with antibodies directed against $\alpha_5\beta_1$ integrin (*middle*) or EGFR (*bottom*). (*A*) (*lanes 1* and *11*) SCC12 cells; (*lane 2*) pcDNA vector transfected cells; (*lanes 3* and *4*) SSIA cells, clones 3 and 6 (NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GM3 ↓) and GT1b ↓), respectively; (*lane 5*) SCC12 cells treated with sense oligodeoxynucleotides to both GalNAcβ1 → 4(NeuAcα2 → 3)Gal β1 → 4Glcβ1-Cer (GM2)/GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GD2) synthase and NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GD3) synthase; (*lane 6*) SCC12 cells treated with antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase (GM3 ↑ and GT1b ↓); (*lane 7*) p17x4-GLVP vector transfected cells; (*lane 8*) GM2/GD2 synthase cDNA transfected cells without RU-486 induction; (*lanes 9* and *10*) GM2/GD2 synthase overexpressing cells, clones 1 and 26 (GT1b ↑); (*lane 12*) DMSO vehicle-treated SCC12 cells; (*lane 13-15*) SCC12 cells treated with GM2; (*lane 13*, GM2 ↑), GM3 (*lane 14*, GM3 ↑), or GT1b (*lane 15*, GT1b ↑). (*B*) SCC12 cells were treated with or without either sense or antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase and the depleted GT1b was "rescued" by treatment with 1 μM GT1b (GM3 ↑ and GT1b ↑ ; *lanes 4–6*) or, as control, with 50 μM GM2 (GM3 ↑ , GT1b ↓ ; *lanes 1–3*) as described in *Materials and Methods*. (*Lanes 1, 4*) SCC12 cells; (*lanes 2, 5*) sense oligodeoxynucleotides-treated SCC12 cells; (*lanes 3, 6)* antisense oligodeoxynucleotides-treated SCC12 cells; (*C*) After SCC12 cells were treated with racemic *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl (PPPP) to deplete gangliosides or with vehicle DMSO as a control, and then specific ganglioside content was "rescued" by incubation with purified ganglioside GM3 or GT1b as described in *Materials and Methods*. (*Lane 1*) SCC12 cells; (*lane 2*) DMSO vehicle-treated SCC12 cells; (*lanes 3–5)* PPPP-treated SCC12 cells without added ganglioside (GM3 ↓ and GT1b ↓ ; *lane 3*) *or with either* GM3 (GM3 ↑ , GT1b ↓ ; *lane 4*) or GT1b (GM3 ↓ , GT1b ↑ ; *lane 5*).

FAK function with FAK antisense oligodeoxynucleotide partially reverses the stimulatory effects of ganglioside depletion on uPA-dependent cell migration (Fig 5) (AG1478-treated *vs* untreated, $p < 0.05$; FAK antisense oligodeoxynucleotide treated *vs* untreated, $p < 0.01$). Treatment with both inhibitors in combination prevents the stimulation of uPA-induced cell migration in cells depleted of gangliosides GT1b and GM3 by sialidase overexpression (Fig 5*B*) (AG1478 + FAK antisense oligodeoxynucleotide-treated *vs* untreated, $p < 0.001$). The migration of cells treated with 0.1% DMSO (control for AG1478 application) or FAK sense oligodeoxynucleotide resembles that of untreated cells (not shown). The extent of cell migration of cells grown in serum-free medium without uPA stimulation is similar to that of cells in which EGFR and FAK signaling is inhibited (not shown).

Discussion

In this report, we have manipulated the expression of gangliosides GT1b and GM3 through both genetic and biochemical means and have studied the specific effects of each ganglioside on uPA-induced migration of SCC12 cells. We have shown that increases in membrane GM3 and GT1b both inhibit uPA-induced cell migration, but that GT1b appears to have a more profound influence. These data suggest that GT1b affects uPA-induced migration at an earlier step than GM3 does. By investigating the mechanism of these actions of gangliosides, we demonstrate distinct roles of gangliosides GT1b and GM3 in regulating uPA-induced motility of squamous carcinoma cells. GT1b prevents the ability of uPAR to complex with $\alpha_5\beta_1$ integrin and subsequently with EGFR, whereas GM3 only inhibits the association of the uPAR/$\alpha_5\beta_1$ integrin complex with EGFR. Thus, GT1b is able to block the direct signaling of uPAR through $\alpha_5\beta_1$ integrin and FAK, as well as EGFR, whereas GM3 is only able to prevent the contribution of EGFR signaling to uPA-induced cell migration (Fig 6). These results further suggest that the formation of an association of uPAR with $\alpha_5\beta_1$ integrin is vital for crosstalk of EGFR with $\alpha_5\beta_1$ integrin in cells exposed to uPA.

The downstream effects of GT1b and GM3 on inhibiting FAK phosphorylation and EGFR phosphorylation are con-

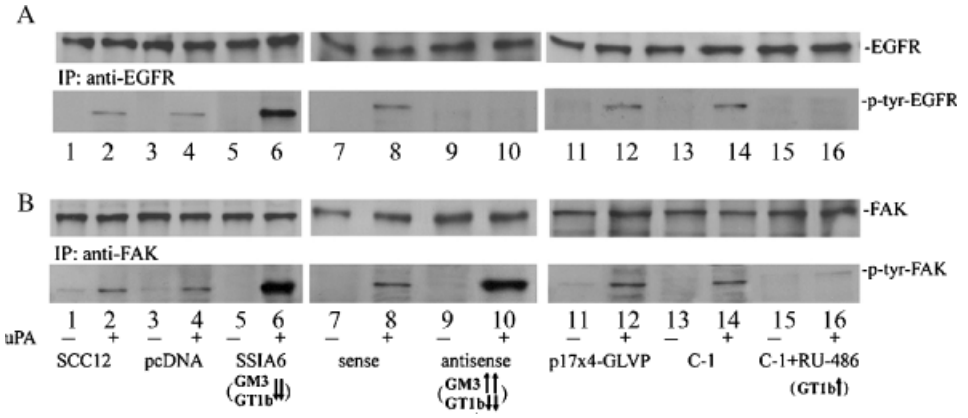

Figure 4
Ganglioside modulation regulates the phosphorylation of both epidermal growth factor (EGFR) and focal adhesion kinase (FAK). Cells were prepared as described in *Material and Methods*. EGFR expression (*top row of A*) and FAK expression (*top row of B*) were detected with anti-EGFR or anti-FAK monoclonal antibody using total protein from the whole-cell lysate. The phosphorylations of EGFR (*bottom row of A*) and FAK (*bottom row of B*) were detected with anti-phosphotyrosine kinase antibody (PY20) using immunoprecipitated EGFR and FAK from the whole-cell lysate.

sistent with the actions of GT1b and GM3 at the membrane level; GT1b is able to block the phosphorylation of both FAK and the EGFR, whereas GM3 solely inhibits EGFR phosphorylation. These effects of GT1b and GM3 are also commensurate with the previously described inhibitory effects of gangliosides on EGFR and FAK signaling in cells when exposed to EGF or grown on a fibronectin matrix (Wang *et al*, 2001a, 2002a, b, 2003a, b). Blockade of EGFR signaling with AG1478 and of FAK signaling through introduction of FAK antisense oligomers are each able to abrogate partially the cell migration induced by ganglioside depletion, whereas the combination of blockade of both EGFR and FAK signaling is able to block migration altogether, reiterating the role of both FAK signaling by $\alpha_5\beta_1$ integrin and EGFR signaling through crosstalk with $\alpha_5\beta_1$ integrin to uPAR signaling-modulated cell migration. These mechanisms likely play a role in the demonstrated ganglioside-induced inhibition of cell migration and tumor malignancy (Kawamura *et al*, 2001; Watanabe *et al*, 2002; Wang *et al*, 2003b).

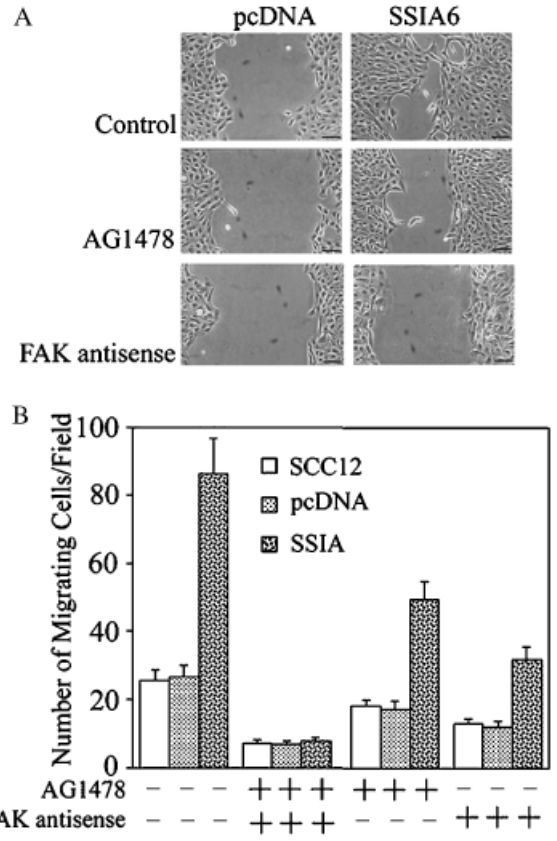

Figure 5
The acceleration of cell migration by ganglioside depletion requires both focal adhesion kinase (FAK) and epidermal growth factor receptor (EGFR) kinase activities. (*A*) Parental SCC12 cells, mock transfected (pcDNA vector transfected) SCC12 cells, or SSIA cells were treated with both AG1478 and FAK antisense oligodeoxynucleotide, or either 250 nM AG1478 or FAK antisense oligodeoxynucleotide as previously described (Wang *et al*, 2002b). 10^8 cells/well were plated onto eight-well cell culture plates and a scratch was made to perform either scratch assay (*A*) or quantitative scratch assay (*B*) as described in *Materials and Methods* (Wang *et al*, 2003b). Starved cells were incubated in Dulbecco's Modified Eagle's Medium/F12 medium containing 10 nM uPA in the absence or presence of inhibitors and/or FAK antisense oligodeoxynucleotide. Migrating cells in the scratched area were counted after incubation for 12 h at 37°C. The extent of migration was quantified as the total number of cells per high-power field with five fields counted, and expressed as the mean number of cells ± SD. *Bar* = 65 μm.

The uPAR, as with other GPI-anchored proteins, tends to be localized to lipid rafts, sphingolipid–cholesterol microdomains on the plasma membrane that concentrate signaling molecules (Harder and Simons, 1997; Horejsi *et al*, 1999). Specifically, uPAR is found in plasma membrane invaginations (caveolae) in association with caveolin-1 (Rothberg *et al*, 1992; Chapman *et al*, 1999; Wei *et al*, 1999), a

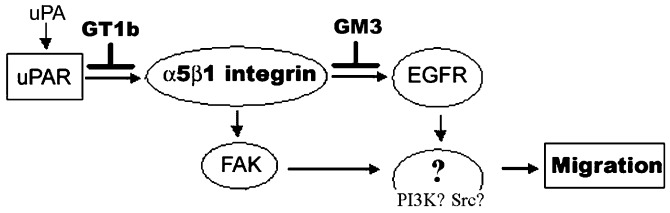

Figure 6
Proposed mechanism of ganglioside effects on urokinase-type plasminogen activator receptor (uPAR) signaling. NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glcβ1-Cer (GT1b) blocks the ability of uPAR to form an association with both $\alpha_5\beta_1$ integrin and the epidermal growth factor receptor (EGFR), thereby blocking cell migration induced by uPAR through inhibition of both focal adhesion kinase (FAK) signaling and, through crosstalk, of EGFR signaling. NeuAcα2 → 3Galβ1 → 4Glcβ1-Cer (GM3) affects the association of uPAR/$\alpha_5\beta_1$ integrin with the EGFR and therefore blocks uPA-induced EGFR signaling that requires crosstalk with uPAR/$\alpha_5\beta_1$ integrin. The inhibitory effects of overexpression of GT1b and GM3 on the activation of phosphatidylinositol-3-kinase and Src kinase may also contribute to the role of specific ganglioside on uPAR signaling-modulated cell migration (Wang *et al*, 2001a, b).

protein that is thought to regulate integrin clustering, a central event in integrin activation (Wary *et al*, 1998). High levels of expression of caveolin-1 enhance the integrin-dependent cell spreading and migration in epithelial tumor cells that is promoted by uPAR signaling activation (Chapman *et al*, 1999), suggesting that caveolin-1 is important for the association of uPAR with other membrane receptors and signaling molecules.

Gangliosides are known to participate in the regulation of cell signaling within lipid rafts (Simons and Ikonen, 1997; Iwabuchi *et al*, 1998; Prinetti *et al*, 1999; Brown and London, 2000; Abrami *et al*, 2001; Brown *et al*, 2002) and/or caveolae (Parton, 1994; Abrami *et al*, 2001) by associating with signaling proteins, such as Src family kinases (Kasahara *et al*, 1997; Kalka *et al*, 2001; Wang *et al*, 2002b), Ras (Parmryd *et al*, 2003), EGFR (Wang *et al*, 2002a), phosphatidylinositol-3-kinase (Wang *et al*, 2002b), integrin (Claas *et al*, 2001), and FAK (Iwabuchi *et al*, 2000; Wang *et al*, 2002b). The demonstration in this study that gangliosides interfere with the association of these receptors at the membrane level suggests that gangliosides may interfere with the role of caveolin-1 in organizing membrane receptors and signaling elements. In fact, endogenous overexpression of specific gangliosides has previously been shown to modify the composition of caveolar signaling domains. For example, overexpression of ganglioside Galβ1 $\rightarrow$ 3GalNAcβ1 $\rightarrow$ 4(NeuAcα2 $\rightarrow$ 3)Galβ1 $\rightarrow$ 4Glcβ1-Cer (GM1) in Swiss 3T3 cells inhibits platelet-derived growth factor receptor phosphorylation, and shifts this growth factor receptor from the "glycosphingolipid-enriched (GEM) domains" with caveolin-1 to the detergent-soluble "non-GEM" domains (Mitsuda *et al*, 2002). Our previous studies have shown that endogenous overexpression of GM3 shifts caveolin-1 itself out of caveolar domains into proximity with the EGFR; given the known inhibitory effect of caveolin-1 on EGFR signaling, this shift could contribute to the inhibitory effect of GM3 on EGFR activation (Wang *et al*, 2002a). This mechanism may also explain the blockade of crosstalk between $\alpha_5\beta_1$ integrin and the EGFR by GM3 if it prevents the physical approximation of the EGFR with $\alpha_5\beta_1$ and uPAR in caveolin-1-rich caveolar domains. The ability of GM3 to bind directly to the EGFR (Wang *et al*, 2001d) and block its direct phosphorylation (Wang *et al*, 2001a, 2002a, 2003a) may be an alternative explanation.

Our previous studies have not found any effect of gangliosides on the expression of signaling molecules, including EGFR (Wang *et al*, 2001a, 2002a, 2003a), FAK (Wang *et al*, 2002b), integrin $\alpha_5\beta_1$ (Wang *et al*, 2001b), and caveolin-1 (Wang *et al*, 2002a). Similarly, we have found no effect of alteration in the expression of gangliosides on either the expression of uPAR or the binding of uPAR with uPA (unpublished data). Our co-immunoprecipitation studies have, however, shown the ability of overexpressed GT1b to associate with both $\alpha5\beta1$ integrin and caveolin-1 in SCC12 cell membrane (unpublished data). In contrast to its inhibitory effect on EGFR signaling, caveolin-1 is known to promote uPAR and $\alpha5\beta1$ integrin signaling within the caveolar domain (Wei *et al*, 1999). Taken together, these data suggest that the mechanism by which GT1b inhibits uPAR signaling may involve either the direct binding of GT1b with $\alpha5\beta1$ integrin (Wang *et al*, 2001b), which prevents the interaction of $\alpha_5\beta_1$ integrin with uPAR or disruption by GT1b of caveolin-1 promoted organization of uPAR, $\alpha5\beta1$ integrin, and FAK within the membrane.

Further investigation should be directed toward elucidating the mechanisms by which specific gangliosides affect uPAR signaling at the membrane level and the downstream signaling elements of uPAR that are impacted by specific gangliosides. The demonstrated effects of ganglioside modulation on uPA-stimulated biologic processes that impact wound healing, inflammatory responses, and cancer metastasis will provide additional impetus to the development of novel therapies that alter membrane ganglioside expression.

Materials and Methods

Cells The human keratinocyte-derived SCC12F2 cell line (SCC12), a generous gift from Dr James Rheinwald (Harvard, Boston, MA), was maintained in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, 1:1, vol/vol) medium (Invitrogen, Carlsbad, California) supplemented with 10% heat-inactivated fetal bovine serum (FBS) without antibiotics in 5% CO_2 at 37°C.

Ganglioside expression modulations Expression of ganglioside was modulated by pharmacological addition of purified ganglioside, treatment with antisense oligodeoxynucleotides or a chemical inhibitor, racemic PPPP (Matreya, Pleasant Gap, Pennsylvania), or stable transfection of genes as a means to metabolize or synthesize specific gangliosides (Table I and Fig 1).

Total depletion of membrane gangliosides Gangliosides were depleted by either overexpression of a human plasma membrane ganglioside-specific *sialidase* cDNA to inactivate cell membrane gangliosides (Fig 1*A*) or treatment of cells with 2 μM of PPPP to block the synthesis of gangliosides (Fig 1*B*). In brief, SCC12 cells were stably transfected with the *sialidase* cDNA (GenBank accession number AB008185, courtesy of Dr T. Miyagi, Tokyo, Japan) (Wada *et al*, 1999) in a pcDNA3 vector using LipofectAMINE reagent. Gene and protein expression in the resultant "SSIA cells" were demonstrated by northern blot and sialidase activity measurements (Wang *et al*, 2001a; Sun *et al*, 2002). PPPP inhibits the activity of glucosylceramide synthase, and thus prevents the formation of glucosylceramide, a precursor for GM3. In contrast to 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), PPPP does not significantly increase ceramide content (Abe *et al*, 1995). Ganglioside depletion was shown by thin-layer chromatography (TLC) immunostaining (Wang *et al*, 2001b, d) and immunofluorescence microscopy (Wang *et al*, 2002a). Four SSIA cell lines (SSIA3, SSIA6, SSIA12, and SSIA25), and two mock transfected pcDNA cell lines were studied.

Overexpression of GM3 by treatment with either antisense oligodeoxynucleotides or pharmacological addition of purified GM3 The cell membrane content of GM3 was endogenously increased as described previously (Wang *et al*, 2002a) by treatment of SCC12 cells concurrently with antisense oligodeoxynucleotides of both GM2/GD2 synthase and GD3 synthase, thereby blocking synthetic pathways downstream of GM3 (Fig 1*C*). GM3 was also increased by incubation of cells (pre-treated without or with PPPP) with 140 μM purified GM3 (Sigma, St Louis, Missouri) for 48 h in DMEM/F12 medium containing 2% FBS as described (Wang *et al*, 2001a, c, 2002b). Ganglioside expression was confirmed using ganglioside ELISA (Wang *et al*, 2002b), TLC immunostaining (Wang *et al*, 2001b, d), and immunofluorescence microscopy (Wang *et al*, 2002a).

Overexpression of GT1b by either stable transfection of GM2/GD2 synthase or pharmacological addition of purified GT1b Because of

anticipated inhibition of SCC12 cell proliferation and decreased cell survival by overexpression of complex gangliosides, particularly GT1b (Wang *et al*, 2001c), GM2/GD2 synthase cDNA (GenBank accession number M83651, courtesy of Dr Lloyd, New York) (Nagata *et al*, 1992) was introduced using an inducible expression system (Wang *et al*, 1994, 1999) that utilizes anti-progestins as inducers (Wang *et al*, 2002b) (Fig 1*D*). Although GM2/GD2 synthase has the capability of driving both "a" pathway (to GM2) and "b" pathway (to GT1b) synthesis, our results showed that overexpression of GM2/GD2 synthase only increases "b" pathway ganglioside in SCC12 cells (Wang *et al*, 2002b). GT1b was also increased by incubation of cells (pre-treated without or with either PPPP or antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase) with 1 μM of purified GT1b for 48h in DMEM/F12 medium containing 2% FBS as described (Wang *et al*, 2001c). Gene expression was detected by northern blotting (Wang *et al*, 2002b) and ganglioside expression was confirmed using ganglioside ELISA (Wang *et al*, 2002b) and TLC immunostaining (Wang *et al*, 2001b, d). Four GM2/GD2 synthase overexpressors (C-1, -5, -11, and -26) and two mock transfected p17x4-tkA/pCEP4 · GL-VP (p17x4-GLVP) cell lines were used in the studies.

Cell migration assays Cell migration assays were performed using both chemotaxis migration assay (Transwell cell culture system, Becton Dickinson, Indianapolis, Indiana) and scratch analysis. For the chemotaxis migration assay, the Transwell inserts with a polycarbonate filter of an 8 μm pore size were pre-coated with 20% FBS on the underside only (Jo *et al*, 2003). After blockade of nonspecific binding sites with 0.5% bovine serum albumin (BSA) for 2 h at 37°C, the insert was placed into a six-well cell culture plate, and the lower portion of the plate was filled with 500 μL serum-free DMEM/F12 medium containing 0.1% BSA and 10 nM uPA (Calbiochem, La Jolla, California). Starved SCC12 cells were pretreated with or without sense or antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase; PPPP; or with purified ganglioside GM3, GT1b or, as a control ganglioside that does not appear to affect SCC12 cell function, GM2 (50 μM) prior to studies. SSIA cell lines, pcDNA3 vector mock transfected SCC12 cells, GM2/GD2 synthase overexpressors, or p17x4-GLVP vector mock transfected SCC12 cells, treated with or without 100 nM of RU-486 inducer for 36 h, were also assessed. These cells were plated onto the upper surface of the filter. Cells were allowed to migrate for 12 h at 37°C in serum-free DMEM/F12 medium containing 0.1% BSA with the continued addition of oligodeoxynucleotides, with or without the RU-486 inducer, PPPP, or purified gangliosides. Cells that migrated into the lower level of the six-well cell culture plate were stained with crystal violet. Results were presented as the mean ± SD. The mean value was acquired by comparing the optical density reading at 560 nm of cells treated with purified gangliosides (GM3, GT1b, or GM2), PPPP, oligodeoxynucleotides, or gene transfected cells induced with or without RU-486 with that of parental SCC12 cells.

To perform scratch assays, cells prepared as indicated above were plated into an eight-well cell culture plate. Cells were allowed to grow in 10% FBS containing DMEM/F12 medium for 4 h, and then were washed with serum-free medium and starved of both serum and growth factors overnight. A 1 mm wide scratch was made across the cell layer using a pipette tip. DMEM/F12 medium with or without 10 nM uPA was added into each well after washing with serum-free medium twice. Plates were photographed after 6, 12, and 24 h. All experiments were performed at least six times.

Quantitative scratch assays were performed to study the effects of kinase inhibitors on ganglioside-modulated changes in migration. Quantitative scratch assays were performed as described above, except that a deep permanent gouge was made with a razor blade into the culture plate at the border of the scratch to allow definitive separation of migrating cells (Wang *et al*, 2003b). Cells were treated without or with 250 nM AG1478 (EGFR kinase inhibitor), or FAK antisense oligodeoxynucleotides (functional blockade of FAK) as described before (Wang *et al*, 2002b). After 6, 12, and 24 h in culture, cells that had migrated into the scratched area were identified and counted. Results were presented as the mean ± SD from five high-power fields per experiment in at least four different experiments.

Incubation of cells with 0.1% DMSO solution was used as vehicle controls for pharmacological addition of purified ganglioside or inhibitor treatment studies; treatment of cells with FAK sense oligodeoxynucleotides was used as a control for FAK antisense oligodeoxynucleotide treatment.

Immunoblotting Immunoblotting was carried out as described (Wang *et al*, 2001b, c) using either total protein from the whole-cell lysate or immunoprecipitated protein, and an enhanced chemiluminescence detection system (Perkin-Elmer Life Sciences, Wellesley, Massachusetts). Monoclonal antibodies directed against phosphotyrosine (PY20), EGFR, FAK (Transduction Laboratories, Lexington Kentucky), $\alpha_5\beta_1$ integrin, or uPAR (Santa Cruz Biotech., Inc. Santa Cruz, California) were applied for immunoblotting studies. If the total protein from the whole-cell lysate was applied for immunoblotting, each blot was re-probed as previously described (Wang *et al*, 2001c) with anti-actin antibody (Santa Cruz Biotech) to confirm equal loading. All blots were repeated in at least three different experiments. The 150 kDa undenatured whole IgG band, 55 kDa denatured heavy chain of the IgG band and, when detectable, the 25 kDa denatured light chain of the IgG band was cut from the blots for figure presentation.

Immunoprecipitation Cells were prepared as indicated above and stimulated with or without 10 nM uPA for 10 min after starvation of serum and EGF overnight. Cells were harvested, lysed in cold immunoprecipitation buffer, and EGFR, FAK, or uPAR was immunoprecipitated from cell lysates as previously described (Wang *et al*, 2001b, c).

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